



Citation for published version:

Taylor, TB & Buckling, A 2013, 'Bacterial motility confers fitness advantage in the presence of phages', *Journal of Evolutionary Biology*, vol. 26, no. 10, pp. 2154-2160. <https://doi.org/10.1111/jeb.12214>

DOI:

[10.1111/jeb.12214](https://doi.org/10.1111/jeb.12214)

Publication date:

2013

Document Version

Publisher's PDF, also known as Version of record

[Link to publication](#)

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SELECTION EXPERIMENTS REVEAL TRADE-OFFS BETWEEN SWIMMING AND TWITCHING MOTILITIES IN *PSEUDOMONAS AERUGINOSA*

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Received February 2, 2011

Accepted May 30, 2011

Data Archived: Dryad doi:10.5061/dryad.rk280

Bacteria possess a range of mechanisms to move in different environments, and these mechanisms have important direct and correlated impacts on the virulence of opportunistic pathogens. Bacteria use two surface organelles to facilitate motility: a single polar flagellum, and type IV pili, enabling swimming in aqueous habitats and twitching along hard surfaces, respectively. Here, we address whether there are trade-offs between these motility mechanisms, and hence whether different environments could select for altered motility. We experimentally evolved initially isogenic *Pseudomonas aeruginosa* under conditions that favored the different types of motility, and found evidence for a trade-off mediated by antagonistic pleiotropy between swimming and twitching. Moreover, changes in motility resulted in correlated changes in other behaviors, including biofilm formation and growth within an insect host. This suggests environmental origins of a particular motile opportunistic pathogen could predictably influence motility and virulence.

KEY WORDS: Experimental evolution, flagella, type IV pili, virulence.

Motility, in a variety of forms, is common within the microbial world. Bacterial motility is presumably a crucial component of fitness in most habitats because it enables cells to move toward sources of nutrition (Wei et al. 2011), move away from potential causes of harm (such as toxins and predation; Chet and Mitchell 1976), and avoid competition with clone mates (Taylor and Buckling 2010). It is also particularly important in host colonization success of opportunistic pathogens (Drake and Montie 1988), and indirectly influences other virulence-related characteristics under strong selection, such as biofilm development (Klausen et al. 2003) and bacteriophage attachment (Schade et al. 1967). Crucially, natural populations of motile bacteria show a large amount of variation in motility even over small spatial

scales (Vos and Velicer 2008), suggesting that different habitats favor different motility strategies (Fux et al. 2005). Understanding the selective forces that act on motility is likely to be important in microbial evolutionary ecology in general, as well as the evolution of virulence of bacterial pathogens. There is a large literature describing associations between virulence and motility (Drake and Montie 1988; Josenhans and Suerbaum 2002; Krukons and DiRita 2003), but we know little about why different motility strategies evolve. Interactions between host and pathogen might play a key role in driving motility and virulence evolution, but in the case of opportunistic pathogens, such as *Pseudomonas aeruginosa*, selection imposed within their natural environment is likely to be crucial in predetermining a

genotype's propensity to cause severe infections (Pallen and Wren 2007).

Bacteria have a range of mechanisms that allow efficient motility under diverse environmental conditions. *Pseudomonas aeruginosa*, arguably the most studied bacteria in terms of motility, expresses two surface organelles that aid motility: a single polar flagellum and retractable polar type IV pili. In addition, it is also able to secrete a lipid-based biosurfactant, rhamnolipids (Mattick 2002). This enables the bacterium to adopt pilus-based twitching on hard surfaces (Henrichsen 1972), flagella-mediated swimming in aqueous environments (Bai et al. 2007), and a coupled pili-flagella action combined with rhamnolipid production known as swarming (comparable to gliding) on semi-soft surfaces (Köhler et al. 2000; Caiazza et al. 2005). The evolution of bacterial motility could be greatly affected by the existence of trade-offs between different motility mechanisms (Stearns 1989), such that an increase in one form of motility necessarily results in a reduction of another (antagonistic pleiotropy; Futuyma and Moreno 1988). The aim of this study is to explore whether such trade-offs exist, and the evolutionary consequences if they do.

There is some evidence to suggest a trade-off between swimming and twitching, although the question has not been extensively addressed. Pili have been shown to be unable to withstand shear forces (Touhami et al. 2006), and this is hypothesized to be important in explaining expression patterns in the highly motile predatory bacteria, *Bdellovibrio bacteriovorus*. *Bdellovibrio bacteriovorus* only extrude pili during prey interactions because, it is thought that fast swimming speeds would be enough to break the pilus fibers from the cell (Evans et al. 2007). Moreover, type IV pili-absent mutants show higher swimming motility in soft agar environments (and vice versa, hyperpilated mutants show reduced motility), suggesting extended pili cause drag and thus inhibit swimming motility (Taylor and Buckling 2010). The possibility of increased twitching motility in the absence of flagella has not been investigated, but data are consistent: flagella-deficient mutants of *P. aeruginosa* retain the ability to twitch (Shrout et al. 2006), and flagella expression is metabolically costly (Soutourina and Bertin 2003). Therefore, one would expect flagella expression to be lost if it is costly to maintain and not required for motility in the selective environment.

To investigate whether a trade-off exists between motility traits in *P. aeruginosa*, we selected for high motility (bacteria that had dispersed the furthest distance on agar plates) in initially isogenic populations under conditions that favored either swimming or twitching motilities. Unlike investigating a possible trade-off using natural isolates, this experimental evolution approach removes the confounding effects of differing genetic backgrounds. A correlated reduction in one type of motility as the other increased would be suggestive of a trade-off mediated by antagonistic pleiotropy, although decay in an unselected trait

may simply arise through genetic drift (Kawecki 1994; Buckling et al. 2007). We therefore set up control lines, where populations were grown in one environment or the other, but there was no selection for high motility: bacteria were selected from random places on agar plates. These control lines would also capture any adaptation to the different agar conditions that might have a correlated effect on motility. Motility was inferred by measuring the dispersal range of the colony over an agar plate after 24 h. Finally, evolved populations were measured for correlated changes in a range of other potentially important traits, growth rate *in vitro* and *in vivo*, and biofilm formation, which can be linked to motility mechanisms.

Materials and Methods

SELECTION REGIME

All lines started with the same genetic background, and changes measured against the ancestral population, therefore differences in motility and virulence are comparable across treatment groups. PAO Δ *mutS*, a mutator strain created via a deletion of the mismatch repair gene *mutS* (Oliver et al. 2004), was used to inoculate 24 starting populations. This strain was not used to gain information regarding the rate, but rather the direction of evolutionary change, and allowed these changes to be observed more quickly. Starting culture for initial plate colonization was grown overnight in shaken (0.9 g) 30-mL glass vials (VWR, Leicestershire, UK) at 37°C, in 6-mL fresh King's B broth (KB: 20 g/L protease peptone, 10 g/L glycerol, 1.5 g/L potassium phosphate, and 1.5 g/L magnesium sulphate heptahydrate). Overnight cultures were vortexed, diluted, and plated to obtain starting densities, and 2.5- μ L pipetted into the agar just below the surface. A total of 24 populations were evolved in one of two environments: 12 in hard KB agar (1.2% wt/vol), which promoted twitching motility; and 12 in liquid KB agar (0.3% wt/vol), which promoted swimming motility. A liquid agar environment was assumed to favor swimming because flagella-absent mutants have been shown to be relatively immobile under these conditions (O'Toole and Kolter 1998a), subsequently, a hard agar environment was assumed to favor twitching, because likewise, pili-absent mutants are relatively immobile (Semmler et al. 1999). All 90-mm-diameter plates (Sterelin, Newport, UK) were poured with 25 mL of KB agar media and were briefly dried in a flow hood for 20 min. Samples were taken daily (approximately 3×10^8 cells) from each of the agar plates using a 1-mL pipette (Finnpipette, Northumberland, UK; for methods see, Taylor and Buckling 2010). After 24 h, in most cases, bacteria grown in liquid agar had reached the edge of the plate. The location from which the sample was taken depended on the selection treatment to which it was assigned. Half the populations were under positive selection for motility where a sample was taken from the edge of

the colony; the other half was under neutral dispersal selection for motility where a sample was taken randomly from the colony using a random number grid. This experimental design resulted in six replicate lines within each test group. Lines were evolved under neutral dispersal selection to capture evolutionary change resulting from adaptation to the different agar conditions, which could have correlated effects on motility, as well as providing insight into the mechanistic basis of the trade-off.

Samples were then placed in a microfuge tube containing 1 mL M9 solution (12.8 g/L Na₂HPO₄, 3 g/L KHPO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl). The pipette tip was washed thoroughly in the solution and the microfuge tube was subsequently vortexed. A total of 2.5 µl was reinoculated onto a new plate (approximately 8×10^5 cells), incubated overnight at 37°C, and the procedure repeated for 15 transfers, after which the samples taken directly from the plate were frozen at -80°C in 20% (wt/wt) KB glycerol.

MOTILITY ASSAYS

Frozen evolved samples and the ancestral strain were used to inoculate 6-mL KB broth. Cultures were left to grow overnight in shaken 30-mL glass vials (0.9 g) at 37°C. Overnight cultures were vortexed and 2.5 µl inoculated onto both hard KB agar (1.2% wt/vol) and liquid KB agar (0.3% wt/vol) to observe differences in motility between the two environments. We used large 140-mm-diameter petri dishes (Sterilin, Newport, UK) to try and capture the maximal distance range of the evolved strains. Area covered by the bacterial colony was measured using Image J 1.42q (<http://rsb.info.nih.gov/ij/docs/faqs.html>) (Abramoff et al. 2004).

IN VITRO GROWTH ASSAYS

To determine the fitness of each individual isolate, and the extent to which growth rate could account for differences between dispersal ranges, growth rate was assayed across ancestral and evolved isolates. Overnight cultures were grown in 6-mL KB at 37°C and diluted in M9 by a factor of 1:100. A total of 10 µl was added to a 96-well flat-bottomed plate containing 90 µL of KB per well. Optical density at 600 nm (OD₆₀₀) was measured every 45 min for 20 h using a microplate spectrophotometer (BioTek Powerwave XS, Northstar Scientific Ltd., Bedfordshire, UK) at an incubation temperature of 37°C. During the period of exponential growth V_{\max} (the rate of change of optical density during log growth) was calculated.

BIOFILM PRODUCTION

To determine the effect of evolutionary environment on biofilm production, ancestral and evolved isolates were grown overnight in 3-mL Luria Broth (LB: 10 g/L Tryptone, 5 g/L Yeast extract, 5 g/L NaCl) shaken 30-mL glass vials at 37°C. OD₆₀₀ was measured and cells were diluted to 0.05 OD in 3-mL fresh LB and grown for 2 h at 37°C in a rotator shaker (0.9 g) to allow

cells to reach exponential growth phase. Cultures were diluted by 1 in 2 with dH₂O and then to 0.025 OD₆₀₀ in 10-mL fresh Tryptone Broth (TB: 10 g/L Tryptone). Into a 96-well flat-bottomed plate, we added 150 µl of each cell culture and left in a humid container at room temperature for 24 h to allow biofilm formation. The plate was washed well in water, and 150 µl of 0.1% crystal violet (CV) solution was added. After mixing, the CV solution was removed and 200 µl of 33% acetic acid solution was added to each well. Finally, 25 µl of dissolved stain was added to 175 µl dH₂O and OD₅₉₅ was measured and recorded: a higher OD reading corresponds to a larger biofilm layer. Methods are adapted from O'Toole and Kolter (1998b), Fletcher (1977), and Jiricny et al. (unpubl. ms.). This method maximizes biofilm production allowing the phenotype to be observed under optimal conditions.

IN VIVO GROWTH ASSAYS

Wax moth larvae, *Galleria mellonella*, have been previously shown to be a good model host for the study of *P. aeruginosa* infections (Jander et al. 2000). Methods were as in Harrison et al. (2006). *Galleria mellonella*, were obtained from Livefood UK (www.livefoods.co.uk, Somerset, UK) and were in their fifth instar at time of inoculation. These were stored in the fridge prior to injection. Overnight KB cultures of ancestral and evolved strains were diluted to 1 in 10⁴ in 0.8% NaCl solution. A total of 100-µl plastic syringes were sterilized and eight larvae were randomly assigned to each of the 13 populations (six populations evolved in hard agar, six populations evolved in liquid agar, and ancestral strain), four were used for controls (injected with 0.8% NaCl). The fresh weight of each larva was recorded prior to injection and the abdomen swabbed with 70% ethanol to prevent contamination. A total of 10 µl of culture was injected into the haemocoel of each larva. Post infection, the larvae were stored in 24-well plates and incubated at 37°C for 8 h. Larvae were then immersed in 70% ethanol to kill surface microbes and placed individually in microcentrifuge tubes containing 1.0 mL 0.8% NaCl solution. Larvae were homogenized by shaking with a sterile ceramic bead (FastPrep[®]-24, MP Biomedicals, Illkirch, France; speed 5.0 m/s for 45 s). Thereafter samples were centrifuged at 1000 rpm for 3 min to pellet hosts' tissues. Twenty micro liter of the homogenate was extracted and diluted to 1 in 10⁴ using 0.8% NaCl. Diluted homogenate was spotted onto KB agar plates supplemented with 15 µg/mL of ampicillin, and incubated overnight at 37°C. The concentration of ampicillin used restricts the growth of natural gut fauna, without impairing *P. aeruginosa* growth (Harrison et al. 2006).

MICROSCOPY

Pictures were taken to visualize differences in colony morphology due to modifications to surface motility organelles, using a Leica

(Buckinghamshire, UK) DM IL LED, with a Leica EC3 digital camera attachment.

STATISTICAL ANALYSIS

Analyses and figures were produced on PASW Statistics 18 (SPSS; part of IBM UK Ltd, Middlesex, UK). Significance of treatment on motility was analyzed using general linear mixed models (GLMMs) (Crawley 2007). Terms used in the model are defined as: (square root) “Dispersal area” [response variable], the area of the bacterial colony after 24-h growth and dispersal relative to the ancestral strain (calculated as area/ancestral area); “Evolutionary environment” [explanatory variable, factor with two levels], the agar type that the strains were evolved in (hard or liquid); “Test environment” [explanatory variable, factor with two levels], the agar type that the strain was grown in during cross-inoculation experiment (hard or liquid); “Selection” (explanatory variable, factor with two levels), the selective regime assigned to the strain (positive or neutral selection for high motility); and, “Replicate line” (nested random factor, within “Selection” and “Evolutionary Environment”). Validity of models was determined by distribution and homogeneity of residuals. Where replicates were measured only once (when looking at correlated effects of selection on nonmotility phenotypes), general linear models (GLMs) (Crawley 2007) using the same factors as above were used. Note that significance levels calculated when multiple tests were carried out were adjusted using sequential Bonferroni correction (Rice 1989).

Results

THE TRADE-OFF BETWEEN TWITCHING AND SWIMMING

To determine whether selection had an effect on motility, evolved strains were cross-inoculated into both the agar type in which these were evolved in and the contrasting agar type (i.e., hard and liquid agar). In all cases, dispersal range was measured relative to the ancestral strain from which these were derived and taken as an estimate of motility (Fig. 1). The average initial area (± 1 SD) of the parental nonevolved strain after 24-h growth and dispersal within each environment was: hard agar, 1.7 cm^2 (± 0.65); and liquid agar, $>185 \text{ cm}^2$. After selection, we found large differences between the dispersal ranges of strains from different evolutionary environments, and these differences were dependent on the agar these were grown in, as well as the selection type these were under (GLMM, three-way interaction between “Evolutionary Environment,” “Test Environment,” and “Selection”; $F_{1,20} = 34.010$, $P < 0.001$). For lines under positive selection for dispersal, we found that motility was relatively greater in the environment in which these were selected (GLMM, interaction between evolutionary and test environments: $F_{1,10} = 320.522$, $P < 0.001$;

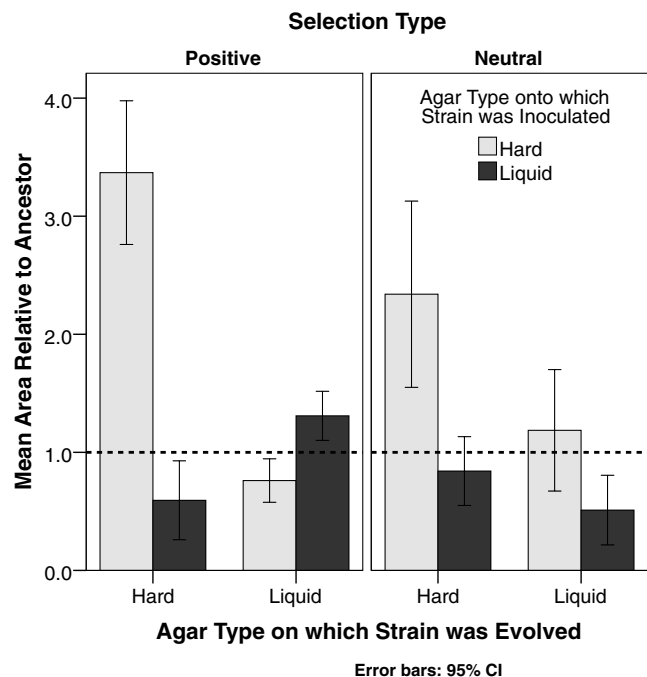


Figure 1. Cross-inoculation of evolved strains between evolutionary environments. The results show the average area of dispersal of the evolved strains (six lines in each treatment group) relative to the ancestral strain from which all strains were derived ($\pm 95\%$ CI). The dashed line indicates a relative dispersal value of 1, that is, when the area of dispersal was equivalent to that of the ancestor.

and strong evidence for a trade-off in the lines under positive selection for dispersal, in that lines showed significant increases (relative to the ancestral strain) in their selective environment, but also showed significant decreases in their nonselective environment (one-sampled t -tests when test environment = evolutionary environment, and test environment \neq evolutionary environment, respectively: under positive selection, $t_{11} = 4.443$, $P = 0.004$, and $t_{11} = 3.644$, $P = 0.012$).

By contrast, there was no tendency for greater dispersal in the evolutionary environment for strains under neutral selection (GLMM, interaction between evolutionary and test environments: $F_{1,10} = 1.520$, $P = 0.250$), demonstrating that selection for motility rather than differences in the agar per se were responsible for the observed changes in lines under positive selection. However considered independently, there was an overall increase in motility in lines under neutral selection evolved and tested on hard agar ($t_5 = 5.097$, $P = 0.004$), and an overall decrease in motility in those evolved and tested on liquid agar under neutral selection ($t_5 = 3.657$, $P = 0.015$).

Our observed trade-off may have resulted from antagonistic pleiotropy (a causal negative genetic correlation between traits), or via the build up of deleterious mutation in the unselected environment, that are neutral in the selective environment (mutation accumulation), or both. If mutation accumulation was important

in driving the trade-off, we would expect to see evidence of mutation accumulation in the neutral selection lines, given that neutral mutations in the evolutionary environment (and hence deleterious mutations in the alternative environment) should be expected to fix regardless of selection for increased motility. This was not the case: the only reduction in motility relative to the ancestor occurred in lines both evolved and measured in liquid agar. These data strongly point to antagonistic pleiotropy driving the swimming-twitching motility trade-off. It is important to acknowledge that within this experimental system, growth and dispersal are intrinsically linked (bacteria that divide faster will have larger dispersal ranges). Here, we assume equal mutation accumulation (which will be a function of elapsed generations) across selective environments. However, this assumption is not necessarily correct, as selecting from a random location (as is the case under neutral selection) would lead to fewer generations, and thus less time for mutations to accumulate, than under positive selection (where cells are transferred from the colony periphery). Crucially, the lines under neutral selection underwent sufficient generations for evolutionary change to be detected, but this evolutionary change was not consistent with mutation accumulation. The observed increase in motility on hard agar in lines under neutral selection for motility in this environment also highlights the role different environments can play in shaping selection.

GROWTH: IN VITRO

Motility is a metabolically costly activity (Ottemann and Miller 1997), and swimming in particular has been documented as an energetic mode of motility (Nandini et al. 2003). We looked for growth differences, both in vitro and in vivo, between strains evolved in the different environments to see whether selection on motility influenced growth patterns. Under in vitro conditions, we found both evolutionary environment (GLM: $F_{1,24} = 15.199$, $P = 0.001$) and selection (GLM: $F_{1,24} = 5.955$, $P = 0.024$) to determine growth rate. However, positive selection for motility reduced the overall growth rate compared to the ancestral strain across both environments (Fig. 2; $t_{11} = -5.557$, $P < 0.001$), and in particular, those evolved in a liquid environment showed lower growth rates (one-sample t -test, liquid evolutionary environment: mean difference = -0.331 , $t_{11} = -5.028$, $P < 0.001$; hard evolutionary environment: mean difference = -0.061 , $t_{11} = -1.689$, $P = 0.199$). Those evolved in hard agar under neutral selection for motility had a growth rate equivalent to the ancestor ($t_5 = 0.836$, $P = 0.441$). In addition, there was a large amount of variation in growth rate between strains that were evolved under neutral selection for motility in a liquid environment (mean = 0.756 , SD = 0.289). Further investigation of all lines revealed a negative correlation between swimming motility and growth rate (data not shown, correlation of growth rate versus dispersal, $\rho = -0.860$, $P = 0.028$).

BIOFILM FORMATION

We expected to see a link between pili-dependent traits and pili use during motility. We chose to examine biofilm formation, a known pili-dependent behavior (Chiang and Burrows 2003). Across all treatments, there was a reduction in biofilm formation relative to ancestral behavior (Fig. 3; $t_{22} = -6.527$, $P < 0.001$). However, the evolutionary environment had only a slight effect on biofilm production (GLM, main effect of evolutionary environment: $F_{1,19} = 4.204$, $P = 0.054$), as did the selection type (GLM, main effect of selection type: $F_{1,19} = 5.105$, $P = 0.036$). Importantly, under positive selection for motility there was a significant effect of evolutionary environment on biofilm formation, with efficient swimmers showing less ability to form stable biofilms ($F_{1,9} = 8.106$, $P = 0.019$), but no effect was detected under neutral selection ($F_{1,10} = 0.388$, $P = 0.547$). Note that difference in degrees of freedom is due to the exclusion of one replicate evolved on hard agar, due to loss of biofilm caused by biofilm disruption.

GROWTH: IN VIVO

Interestingly, growth rates within the *G. mellonella* host do not mirror growth rates in vitro (Pearson's correlation: $r = 0.37$, $P = 0.086$). We found evolutionary environment to be a strong determinant for growth in vivo (Fig. 4; GLM, main effect of evolutionary environment: $F_{1,20} = 5.876$, $P = 0.025$), as was the selection type (GLM, main effect of selection: $F_{1,20} = 5.086$, $P = 0.035$). Within a host, strains under positive selection and evolved in a hard agar environment showed higher growth than those evolved in liquid agar ($t_{22} = 2.886$, $P = 0.009$). Specifically, those evolved on a hard agar environment grew within the host to a density equivalent to the ancestor ($t_{11} = 2.013$, $P = 0.069$), but those evolved on liquid agar grew significantly less ($t_{11} = -2.835$, $P = 0.016$). Under neutral selection, there were no differences in growth within a host between those strains evolved in a hard or liquid agar environment ($t_{22} = 1.741$, $P = 0.096$). However, both had lower growth rates than the ancestral strain (one-sample t -test: on hard agar, $t_{11} = -2.697$, $P = 0.021$; on liquid agar, $t_{11} = -8.319$, $P < 0.001$).

OBSERVATIONAL MORPHOLOGY DIFFERENCES

There was a dramatic change in colony morphology over the course of the experiment (Fig. 5). Bacterial changes in physiology and morphology can be caused both directly, by physical contact to the growth surface, and indirectly, via cell to cell signalling that provides information regarding cell proximity (Harshey 2003). On hard agar, the edge of the colony became less defined, and magnification revealed elaborate colony edges (Fig. 5C), presumably caused by increased production of extracellular products (e.g., rhamnolipids). On liquid agar, these strains had comparably smaller colonies and magnification showed large waves that suggest periods of density-dependent motility (Fig. 5D;

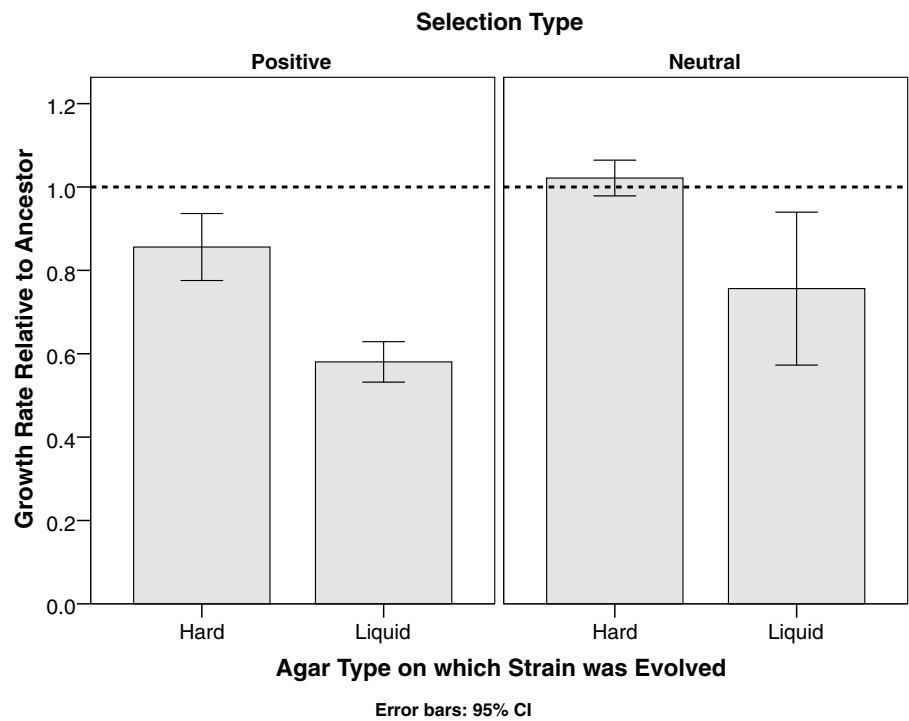


Figure 2. Average growth rate of evolved strains in vitro relative to the ancestral strain ($\pm 95\%$ CI). The dashed line indicates a relative growth rate equivalent to that of the ancestor.

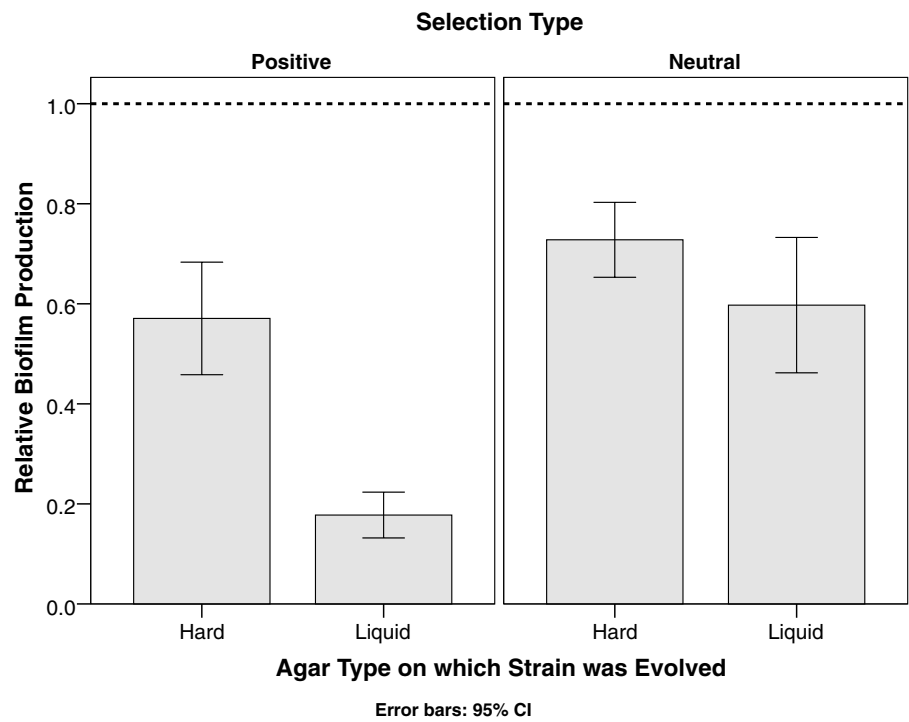


Figure 3. Biofilm production of evolved strains. Results show the average biofilm production of six isolates evolved in each experimental environment, relative to the ancestral strain from which all evolved strains were derived ($\pm 95\%$ CI). The dashed line indicates a relative level of biofilm production equivalent to that of the ancestor.

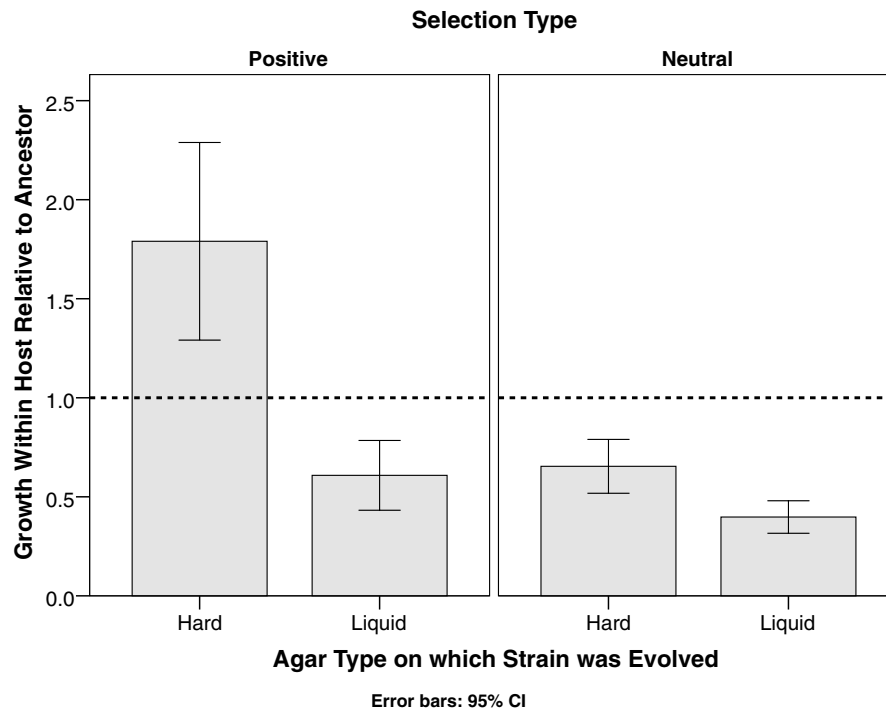


Figure 4. Average growth rate of evolved strains within a host (*G. mellonella*) relative to the ancestral strain. The dashed line indicates a relative growth rate equivalent to that of the ancestor.

Harshey 2003). Those evolved on liquid agar, showed small, compact colonies with smooth edges when grown on hard agar (Fig. 5E). However, on liquid agar, colonies were much larger and under magnification cells appeared to be moving in a directional manner, with small areas of active cells at the edges suggesting apparent coordinated motility (Fig. 5F).

Discussion

We investigated trade-offs between motility mechanisms in *P. aeruginosa* by experimentally selecting lines for increased dispersal in environments that favored swimming and twitching. We found evidence for a trade-off between the motility mechanisms: strains that were selected for increased motility in a hard agar environment lost swimming motility relative to the ancestral strain; and conversely, those that were evolved in a liquid agar environment were poor at twitching (Fig. 1). We found no evidence that the environment per se was responsible for this pattern, because lines evolved in the same environments but with no selection for increased motility, did not display this trade-off. We suggest this trade-off largely arises because of antagonistic pleiotropy, whereby improved performance in swimming necessarily reduces twitching performance. It is possible that the build-up of mutations that are neutral in the selective environment but deleterious in the alternative environment (mutation accumulation; Kawecki 1994) contributed to this pattern. However, the absence of con-

sistent reduction in motility in the unselected environment when bacteria underwent neutral selection for motility suggests that this is unlikely.

To determine whether the differences observed in dispersal range could be explained to some extent by differences in growth rate, and whether changes in motility might carry metabolic costs, we tested the growth rate (V_{max}) of evolved relative to ancestral strains (Fig. 2). Surprisingly, in vitro maximal growth rate decreased in all lines relative to the ancestor, presumably because maximal growth rate is not an important determinant of fitness under these experimental conditions. This suggests that growth rate per se cannot explain patterns of dispersal. However, we did find that growth rate was most reduced in lines evolved in liquid, and within the liquid evolved lines there was a negative correlation between growth rate and swimming dispersal: bacteria were efficient swimmers and slow growers, or poor swimmers and fast growers.

To understand how a trade-off between swimming and twitching might indirectly influence other important life-history traits, aside from motility, we looked at two essential bacterial functions: (1) biofilm formation (a trait vital for pathogenicity, antibiotic resistance, biofouling, and waste water treatment (Costerton et al. 2005; Kolter and Greenberg 2006; Xavier and Foster 2007); and, (2) growth within a host (to determine how these changes might particularly affect a pathogenic lifestyle). First, type IV pili, which are used for twitching motility, mediate

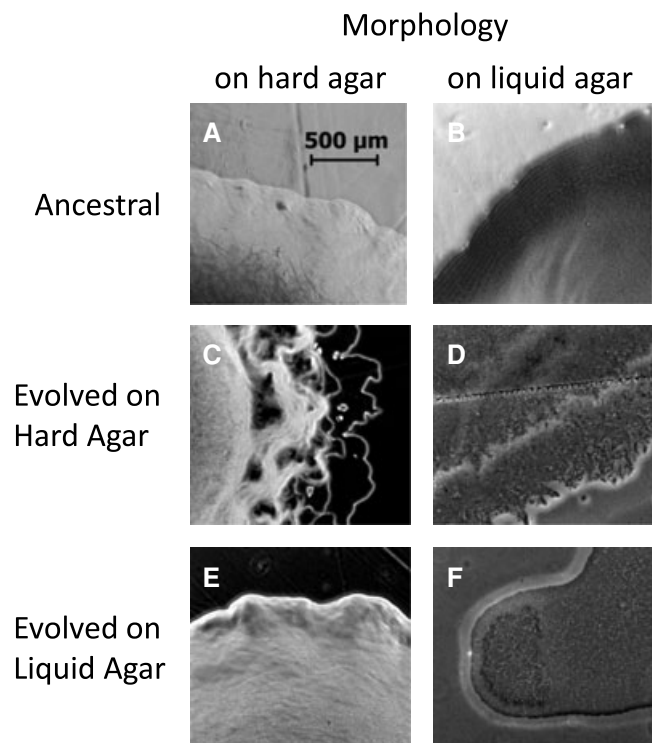


Figure 5. Photographs of agar plates showing colony edge morphology (Leica DM IL LED, with a Leica EC3 digital camera attachment). The top row shows the morphology of ancestral strains as seen on hard and liquid agar, respectively (A and B); the second row shows strains that were evolved on a hard agar environment, as seen when grown on hard agar (C), and liquid agar (D); the third row shows strains that evolved in liquid agar, as seen when grown on hard agar (E), and liquid agar (F).

cell interactions between surfaces and other cells, and hence also influence biofilm formation (Chiang and Burrows 2003; Strom and Lory 1993; Jenkins et al. 2005; Barken et al. 2008). Surprisingly, we found reduced biofilm formation relative to the ancestor in all of the evolved lines (Fig. 3), possibly because flagella, which mediate swimming motility, are also important factors in biofilm formation (Verstraeten et al. 2008). However, when only evolved lines were considered, there was, as expected, a positive correlation between twitching motility and biofilm formation, with greater loss of biofilm formation found in evolved lines that had reduced twitching motility. Second, both flagella and pili have proved to be important in establishing an infection (Tang et al. 1995; Feldman et al. 1998), it was therefore unclear how selection for different swimming motilities would affect *in vivo* growth. We assayed bacterial growth rate (an indicator of bacterial virulence; Harrison et al. 2006; Inglis et al. 2009; Racey et al. 2009) over 8 h of infection of the wax moth larvae (*G. mellonella*) and found that both neutral selection treatments and selection for swimming motility reduced *in vivo* growth relative to the ancestor, whereas growth increased by more than 50% relative to the ancestor in lines

selected for increased twitching motility. This variation was not directly correlated with growth rates *in vitro*. These data suggest that twitching motility may positively correlate with virulence, a result consistent with previous studies showing greatly reduced virulence of type IV pili mutants which are unable to twitch (Cocoli et al. 1999; Lyczak et al. 2000; Jenkins et al. 2005).

Our data suggest a link between environmental selection pressures and virulence of *P. aeruginosa*: a potentially important finding given that a majority of cystic fibrosis patients are believed to be typically colonized by strains of environmental origin (Römling et al. 1994, 1997; Kiewitz and Tümmler 2000; Spencer et al. 2003; Mathee et al. 2008). Consistent with this view, many studies have investigated differences between environmental and clinical strains, and found a large amount of conservation of genes across the isolates (Römling et al. 1994, 1997; Spencer et al. 2003; Wolfgang et al. 2003; Head and Yu 2004; Mathee et al. 2008). This suggests that selection for maintenance of such traits does exist in environmental reservoirs (Wolfgang et al. 2003), however, environmental isolates tended to be lumped together, and the possible link between motility phenotype and the specific environmental origin has not been investigated.

We also found the agar environment to be a determinant of motility, independent of selective treatment. Motility increased in all strains evolved in a hard agar environment, regardless of whether we imposed positive or neutral selection for motility. This is likely to be due to the distribution and diffusibility of nutrients within the environment. The viscosity of the environment will determine the diffusion of nutrients (and microbial excreted products) (Allison 2005), therefore within a highly viscous patch, when nutrients are used up these will take longer to be replenished. This will increase competition for resources, and an individual will gain a fitness benefit if it is able to escape competition by moving to unoccupied patches (Wei et al. 2011). This benefit will be greater in largely clonal populations (as is the case here) due to the indirect fitness benefits gained from alleviating competition between kin (Hamilton and May 1977; Rousset and Gandon 2002; Taylor and Buckling 2010). In contrast, we found swimming motility to increase only under positive selection for high motility, and decrease in the absence of selection, regardless of environment. There are two potential causes of this, if swimming is essentially a neutral trait in these environments it might decay by random drift (Hall and Colegrave 2008), alternatively, if it is costly to maintain and perform, the bacteria might gain a selective advantage by loss of function (Velicer et al. 1998).

This study shows that environment plays a crucial role in the evolution of bacterial motility, and that fitness in specific environments may be limited by trade-offs between motility traits and linked phenotypic characters. Despite the simplicity of our experimental environments, these trade-offs are likely to hold

in natural settings. Indeed, trade-offs may well be stronger in more stressful natural environments, in the presence of lower nutrients, greater competition, and natural enemies. This trade-off has the potential to limit the ubiquity of particular strains, diversify populations, and alter within-host behavior.

ACKNOWLEDGMENTS

We thank the European Research Council and the Leverhulme Trust for funding as well as H. Leggett and F. Harrison for laboratory support.

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Associate Editor: A. Read